Association of serum calcium with serum sex steroid hormones in men in NHANES III

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Abstract: BACKGROUND: Bone is a positive regulator of male fertility, which indicates a link between regulation of bone remodeling and reproduction or more specifically a link between calcium and androgens. This possibly suggests how calcium is linked to prostate cancer development through its link with the reproductive system. We studied serum calcium and sex steroid hormones in the Third National Health and Nutrition Examination Survey (NHANES III). METHODS: Serum calcium and sex steroid hormones were measured for 1262 men in NHANES III. We calculated multivariable-adjusted geometric means of serum concentrations of total and estimated free testosterone and estradiol, androstanediol glucuronide (AAG), and sex hormone binding globulin (SHBG) by categories of calcium (lowest 5% [<1.16 mmol/L], mid 90%, top 5% [1.30 mmol/L]). RESULTS: Levels of total and free testosterone, total estradiol or AAG did not differ across categories of serum calcium. Adjusted SHBG concentrations were 36.4 for the bottom 5%, 34.2 for the mid 90% and 38.9 nmol/L for the top 5% of serum calcium (Ptrend = 0.006), free estradiol levels were 0.88, 0.92 and 0.80 pg/ml (Ptrend = 0.048). CONCLUSIONS: This link between calcium and sex steroid hormones, in particular the U-shaped pattern with SHBG, may, in part, explain why observational studies have found a link between serum calcium and risk of prostate cancer.

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Association of serum calcium with serum sex steroid hormones in men in NHANES III

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Abstract

Background: Bone is a positive regulator of male fertility, which indicates a link between regulation of bone remodeling and reproduction or more specifically a link between calcium and androgens. This possibly suggests how calcium is linked to prostate cancer development through its link with the reproductive system. We studied serum calcium and sex steroid hormones in the Third National Health and Nutrition Examination Survey (NHANES III).

Methods: Serum calcium and sex steroid hormones were measured for 1,262 men in NHANES III. We calculated multivariable-adjusted geometric means of serum concentrations of total and estimated free testosterone and estradiol, androstanediol glucuronide (AAG), and sex hormone binding globulin (SHBG) by categories of calcium (lowest 5% [<1.16 mmol/L], mid 90%, top 5% [e 1.30 mmol/L]).

Results: Levels of total and free testosterone, total estradiol, or AAG did not differ across categories of serum calcium. Adjusted SHBG concentrations were 36.4 for the bottom 5%, 34.2 for the mid 90%, and 38.9 nmol/L for the top 5% of serum calcium (P_{trend}=0.006), free estradiol levels were 0.88, 0.92, and 0.80 pg/ml (P_{trend}=0.048).

Conclusions: This link between calcium and sex steroid hormones, in particular the U-shaped pattern with SHBG, may, in part, explain why observational studies have found a link between serum calcium and risk of prostate cancer.

Introduction

Following positive findings for an association of intake of dairy products and calcium supplements with risk of prostate cancer (1-2), especially advanced disease, the link between serum levels of calcium and prostate cancer has been investigated lately (3-8). In the National Health and Nutrition Examination Survey I (NHANES I), comparing men in the top with men in the bottom tertile of serum calcium, the relative hazard was 2.68 (95% CI: 1.02-6.99) for fatal prostate cancer and 1.31 (95% CI: 0.77-2.20) for incident prostate cancer for men in the highest tertile of serum calcium compared to the lowest (4). Moreover, a statistically significant correlation was found between serum calcium and free prostate-specific antigen (PSA) in NHANES 2005 to 2006, which indicates a possible link between the calcium metabolism and prostate cancer etiology (5).

The link between calcium metabolism and PCa has also been found in pre-clinical studies. It was for instance shown in an athymic mouse model that tumour growth was effected by changes in dietary intake of vitamin D and calcium (9) . When trying to identify the 17alpha-hydroxylase/17,20 lyase inhibitor's (VN/124-1) mechanism of action against androgen-dependent cancer models, it was shown that this drug affects intracellular calcium levels and as a consequence the release of calcium from the endoplasmic reticulum (10). This is again another illustration of a potential link between the calcium and androgen metabolism.

Recently it was also shown that bone is a positive regulator of male fertility, which suggests a link between regulation of bone remodeling, energy metabolism, and reproduction or thus a link between the calcium and androgen metabolism (11). Since androgens promote cell proliferation and inhibit prostate cell death it is plausible that calcium is related to the risk of prostate cancer development via its link with the reproductive system (12-14). Androgens have been shown to modulate calcium through direct regulation of the *STIM1* gene by androgen receptor binding to the *STIM1* promoter (15). In relation to cancer, it has also been thought that there is a link between estrogen and calcium sensing receptor genotypes and serum calcium (16). The estrogen receptor-alpha, vitamin D receptor,

and the calcium-sensing receptor have been linked to carcinogenesis through their effects on calcium levels (16-17).

In a cross-sectional analysis, we investigated serum calcium concentration in relation to concentrations of total testosterone, total estradiol, sex hormone-binding globulin (SHBG), androstanediol glucuronide (AAG), free testosterone, and free estradiol in adult males in the NHANES III, a nationally representative sample of non-institutionalized Americans. We hypothesized that men with higher serum calcium would have higher testosterone levels, lower estradiol, and higher SHBG.

Methods

Study population

The National Center for Health Statistics (NCHS) conducted NHANES III between 1988 and 1994 (18) and designed it as a multistage stratified, clustered probability sample of the US civilian non-institutionalized population who was at least two months old. All subjects participated in an interview conducted at home and an extensive physical examination, which included a blood sample performed at a mobile examination center (18). NHANES III was conducted in two phases (1988-1991 and 1991-1994) which both lead to independent unbiased national estimates of health and nutrition characteristics. Within each phase, subjects were randomly assigned to participate in either the morning or afternoon/evening examination session. Of the 2,205 men, who participated in the morning session of Phase I (1988-1991), we selected all men aged 20+ years who had serum measurements for total testosterone, total estradiol, SHBG, AAG, and calcium (n=1,262).

Hormone measurements

Stored serum samples were assayed for sex steroid hormones at the Children's Hospital Boston, MA. Testosterone, estradiol, and SHBG concentrations were measured with competitive electrochemiluminescence immunoassays on the 2010 Elecsys autoanalyzer (Roche Diagnostics,

Indianapolis, IN) in 2005. AAG, an indicator of the conversion of testosterone to dihydrotestosterone, was measured with an enzyme immunoassay (Diagnostic Systems Laboratories, Webster, TX). Laboratory technicians were blinded to participant characteristics. The detection limits of the assays were 0.02 ng/ml, 5 pg/ml, 0.33 ng/ml, and 3 nmol/l for testosterone, estradiol, AAG, and SHBG, respectively. The coefficients of variation for quality control specimens were: testosterone 5.9% and 5.8% at 2.5 and 5.5 ng/ml, respectively; estradiol 2.5%, 6.5%, and 6.7% at 39.4, 102.7 and 474.1 pg/ml, respectively; AAG 9.5% and 5.0% at 2.9 and 10.1 ng/ml, respectively; and SHBG 5.3% and 5.9% at 5.3 and 16.6 nmol/l, respectively. Quality control samples with a mean estradiol concentration of 39.4 pg/nL were also performed, which is then range of the of typical male estradiol concentrations (interassay coefficient of variantion: 2.5%)(19). Free testosterone was estimated from total testosterone, SHBG, and albumin and free estradiol was estimated from total estradiol, SHBG and albumin using mass action equations (20-21).

Exposure measurements

Information on age, race/ethnicity, cigarette smoking, alcohol consumption, and physical activity was collected during the interview. Race and ethnicity were combined into four racial/ethnic groups: non-Hispanic white, non-Hispanic black, Mexican American and other. Participants were classified as never, former, and current smokers (<20, 20-40, e40 cigarettes per day) based on the self-reported smoking habits. Frequency of alcohol consumption was measured by a food frequency questionnaire and categorized by times per week. Vigorous physical activity was defined by the following activities: jogging or running; swimming or aerobics (for men 40 years or older); biking, dancing, gardening, and calisthenics (for men 65 years or older); and walking and lifting weights (for men 80 years and older). Participants were defined as being diabetic when they reported a diagnosis of diabetes or when they were using insulin or diabetic medication. Percent body fat was estimated from anthropometric and bioelectrical impedance data using the equations of Chumlea and colleagues (22). Serum calcium was measured using a Hitachi 737 Analyzer (Boehringer Mannheim Diagnostics, Indianapolis, IN) (23-24). 25-hydroxy vitamin D was measured using the Diasorin radioimmunoassay kit (Diasorin,

Stillwater, MN, USA) on frozen serum from 1994 to 1995. Coefficients of variations from quality control samples ranged from 13% to 19%. The radioimmunoassay kit was calibrated using high-performance liquid chromatographically purified vitamin D every six months. The protocols for the conduct of NHANES III were approved by the institutional review board of the NCHS, Centers for Disease Control and Prevention. Written informed consent was obtained from all participants. The Institutional Review Boards at the Johns Hopkins Bloomberg School of Public Health and the NCHS, Centers for Disease Control and Prevention approved the assay of stored serum specimens for the Hormone Demonstration Program.

Statistical Analysis

All analyses were conducted with Statistical Analysis Systems (SAS) release 9.2 (SAS Institute, Cary, NC) and SUDAAN 9.0 software (Research Triangle Park, NC) as implemented in SAS 9.2. Phase I morning sampling weights for NHANES III were used to account for sampling variability and to adjust for differential probability of selection of persons (18). First, we calculated the age-adjusted means or percentages of characteristics of the men by categories of serum calcium concentration by adjusting for the age distribution of the US population according to the 2000 Census. Since the calcium metabolism is tightly controlled, the following categories of calcium were chosen to capture extreme variation: the bottom 5%, mid 90%, and top 5%. Next, we calculated crude and adjusted geometric mean concentrations of the sex steroid hormones and their 95% confidence intervals (CI) by categories of serum calcium using linear regression. Hormone concentrations were not normally distributed, so we transformed them using the natural logarithm. The multivariable model was adjusted for age (continuous), race/ethnicity and factors that have been associated with hormone concentrations in previous NHANES III analyses: percent body fat, vigorous physical activity (yes or no), serum levels of vitamin D, cigarette smoking (never, former, current), alcohol intake (<2, 2-3, 4-6 times a week, or daily or more), and history of diabetes. We also adjusted for serum levels of albumin to obtain an estimate of the free ionized calcium levels, which is the amount of metabolically active serum calcium (25). In a sensitivity analysis, we performed an additional adjustment for comorbidity as both sex steroid hormones and calcium may be associated with other comorbidities. The

comorbidity was evaluated with a comorbidity coefficient similar to the Charlson Comorbidity Index, as used in other NHANES III-based analyses (26). Each of the comorbidities available in the dataset contributed one point to the composite index with additional points given for older age. In addition, we performed stratified analyses for age groups (20-39, 40-59, e 60 years), racial/ethnic groups, and serum levels of vitamin D (< / e 24.2 ng/mL) to determine whether the results were consistent between younger and older men, between different racial/ethnic groups, and between men with different vitamin D levels. Since older men, men of African descent, and men with lower vitamin D levels tend to have a higher risk of prostate cancer (27-28), it is possible that the association between calcium and sex-steroid hormones differs for these groups. Since vitamin D is regulated by calcium and has been shown to have antiproliferative effects on human prostate cancer cells (5, 29), the above analyses were also performed for serum calcium by low and high serum levels of vitamin D (30). We performed a test for interaction for all the above stratified analyses by introducing an interaction term in the linear regression models and testing its coefficient by the Wald test.

Results

Baseline characteristics of the study population by categories of serum calcium are shown in Table 1. The distribution of age, race/ethnicity, % body fat, and serum albumin generally did not differ statistically by categories of calcium. Those in the lowest 5% of serum calcium concentration had the largest proportion of current smokers (44.1%). Those in the highest 5% of serum calcium had the largest proportion of men who performed vigorous physical activity (18.1%). The mean level of vitamin D (26.3 ng/mL) was lowest for those in the bottom 5% of serum calcium. The mean level of albumin was slightly higher (43.7 g/L) in those in the top 5% of serum calcium. Finally, vitamin D concentration increased across and categories of serum calcium (P=0.038).

When comparing the fully adjusted geometric means of the sex steroid hormones by categories of serum normalized calcium, levels of total and free testosterone, total estradiol, or AAG did not differ across categories of serum calcium. However, there was an upside down U-shaped association

between categories of serum calcium and free estradiol: 0.88 for the bottom 5% of serum calcium, 0.92 in the middle 90%, and 0.80 pg/mL in the top 5% (Table 2). In addition, there was a U-shaped association for SHBG (P=0.006) (Table 2) across categories of serum calcium: 36.4 for the bottom 5% of serum calcium, 34.2 in the middle 90%, and 38.9 nmol/mL for the top 5%. Additional adjustment for comorbidity did not alter the results dramatically (results not shown).

Although age was not a statistically significant effect modifier, Table 3 shows that the above patterns for mean SHBG and free estradiol by serum calcium levels were only observed in men aged <40 (P-trend=0.003 and 0.092, respectively).

When stratifying by race/ethnicity, effect modification was observed for SHBG, total and free estradiol ($P_{interaction}$=0.030, 0.005, and 0.003, respectively) (Table 4). Interestingly, the U-shaped pattern for SHBG was only observed for non-Hispanic white men, whereas an upside-down U-shaped pattern was observed among Mexican American men and a positive association was seen for non-Hispanic black men (Table 4).

No statistically significant effect modification was observed by median 25-hydroxy vitamin D levels, but the above observed U-shaped association for SHBG was only apparent for men with vitamin D levels ≥ 24.2 ng/mL, whereas the association for total estradiol was only apparent among those with vitamin D levels < 24.2 ng/mL. A sensitivity analysis including only non-Hispanic white men, did not alter these findings (results not shown).

Serum calcium level modeled as a continuous measure was not statistically significantly associated with any of the hormones.

Discussion

This cross-sectional study investigated how serum levels of calcium are correlated with levels of sex steroid hormones. We found that circulating calcium was especially associated with circulating levels of SHBG and free estradiol, but not total and free T, total E2, or AAG. This association was, in particular, observed in men aged 20 to 40 years and non-Hispanic white men. When stratified by levels of vitamin D, the U-shaped pattern for SHBG was only observed among men with 25-hydroxyvitamin D levels e24.2 ng/mL, whereas the upside-down U-shaped pattern for free estradiol was only observed among men with 25-hydroxyvitamin D levels <24.2 ng/mL – however, the interaction was not statistically significant.

Several observations and pathways suggest a link between serum calcium levels and sex steroid hormones (31-32): (1) both sex steroid hormones as well as serum calcium levels have been found predictive for the risk of prostate cancer development (27, 33); (2) sex steroid hormones and serum calcium levels have also been connected with bone health (Figure 1).

Firstly, the link between sex steroid hormones and prostate cancer has been addressed in the context of androgen deprivation therapy and illustrated that the prostate is both an androgen-dependent and androgen-sensitive organ (34). Several epidemiological studies also assessed the link between serum levels of calcium and risk of prostate cancer (3-4, 6-7). Moreover, a study by Berry and colleagues showed that androgens modulate calcium through the direct regulation of the STIM1 gene by androgen receptor binding to the STIM1 promoter (15). However, currently no epidemiological study has examined whether an interaction in the association between circulating calcium levels and sex steroid hormone levels on the risk of prostate cancer incidence and progression may exist. Our data show an association between circulating levels of serum calcium and SHBG and free estradiol. Both sex steroid hormones have been found to be associated with severity and progression of prostate cancer (35-37). Using 18 prospective studies that included 3886 men with incident prostate cancer, the Endogenous Hormones and Prostate cancer Collaborative Group found an inverse association

between serum concentration of SHBG prostate cancer risk (RR in the highest versus lowest fifth: 0.86; 95% CI: 0.75-0.98)(38). In the current study, the U-shaped association for SHBG was in particular observed for men aged 20 to 40 years and non-Hispanic white men, but there was also a statistically significant but different pattern in Mexican American men. This U-shaped pattern between levels of SHBG and serum calcium is also consistent with the U-shaped pattern for serum levels of calcium and risk of prostate cancer previously found (8). A modifying effect of race/ethnicity was also observed for the association between free estradiol and serum calcium. This is especially interesting since it has been previously shown that testosterone concentrations did not differ notably between black and white men in NHANES III (19).

Another possible pathway between calcium and prostate cancer risk is via vitamin D, which is regulated by calcium and has been shown to have antiproliferative effects on human prostate cancer cells (5, 29). It has recently also been highlighted how calcium/calmodulin-dependent protein kinase kinase 2 (CAMKK2) is a key effector of the androgen receptor, regulating glycolytic flux by activating AMPK-PFK (5' AMP-activated protein kinase - phosphofructokinase) signaling, which in turn drives anabolism and thereby controls prostate cancer cell proliferation and tumour growth. CAMKK2 is thus regulated by the calcium/calmodulin complex and suggested to be an androgen receptor target in both androgen-dependent and castrate-resistant prostate cancer (32). Although there was no statistically significant interaction, the pattern for SHBG (i.e., low levels of SHBG with low and high calcium concentration and higher levels among men with calcium levels in the normal range) was only observed among men with 25-hydroxyvitamin D levels e24.2 ng/mL, whereas there was no association between circulating levels of SHBG and calcium in men with 25-hydroxyvitamin D levels <24.2 ng/mL. In contrast, the pattern for free estradiol (i.e., low free estradiol concentrations among men in the bottom and top categories of serum calcium) was only observed among men with 25-hydroxyvitamin D levels <24.2 ng/mL.

Secondly, sex steroids have been found to influence bone mass in healthy men. Bone mineral density has been positively correlated with estradiol levels, whereas a inverse correlation was found with sex hormone-binding globulin, and no correlation was found with testosterone in men older than 65 years of age (31). It is also suggested that bone resorption is mainly regulated by estradiol, with a smaller but independent contribution of testosterone, whereas bone formation is regulated in equal proportions by the two hormones (31). Moreover, osteoporosis is a common side effect following androgen deprivation therapy for men with prostate cancer (39). It is observed that a rapid loss of bone-mineral density occurs within the first six to twelve months of endocrine treatment (40-42). A survival analysis based on the US SEER program, found that among men surviving at least five years after diagnosis, 19.4% of those who received androgen deprivation therapy had a fracture, as compared with 12.6% of those not receiving endocrine treatment (P<0.001)(43). It was recently shown in the Osteoporotic Fractures in Men Study that adverse skeletal effects of low sex steroid levels (SHBG, free testosterone and estradiol) were more pronounced in men with low vitamin D levels (44). Overall, our results showed an inverse association between circulating levels of free estradiol and calcium, but when stratified by levels of vitamin D this association was only apparent among men with low vitamin D levels. Nevertheless, it is also important to point out that free estradiol was calculated based on levels of SHBG, total estradiol, and albumin. Therefore, it is possible that the associations observed for free estradiol may follow from the patterns observed for SHBG.

This study has several strengths including its generalizibility following the use of nationally representative data. Therefore it was also possible in our analysis to perform a stratified analysis by race/ethnicity. We were able to adjust for many potential confounding factors and examine effect modification by age, race/ethnicity, and vitamin D levels. A limitation of this study is that it relies on one single measurement so that it may be prone to measurement error and within-person variation. Moreover, our study used immunoassays instead of mass spectometry to evaluate serum levels of sex steroid hormones. Repeated measurements may strengthen the accuracy of the observed associations.

Conclusion

The present results suggest that there may be a positive association between sex steroid hormones and serum levels of calcium. This link between calcium and sex steroid hormones, in particular SHBG, may explain why observational studies have found a link between serum calcium and risk of prostate cancer. The observed U-shaped pattern for SHBG and serum calcium was consistent with the U-shaped pattern for serum calcium and prostate cancer risk found in other studies. A prospective study evaluating both serum levels of sex steroid hormones and calcium linked to risk and severity of prostate cancer or fractures could provide more insight into their roles related to prostate tumorigenesis and bone health.

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Tables

	Serum normalized total calcium (mmol/L)		
	Lowest 5% **<1.16**	**Mid 90%** **1.16-1.30**	**Top 5%** **e1.30**
n	47	1137	78
Age (years)			
Mean (SE)	45.19 (0.83)	45.69 (0.25)	46.14 (1.11)
Race - Ethnicity (%)			
Non-Hispanic white	78.80	78.01	72.72
Non-Hispanic black	10.31	9.01	18.61
Mexican-American	5.82	4.54	5.93
Other	5.07	8.44	2.74
Percent Body fat (%)			
Mean (SE)	25.21 (1.50)	25.27 (0.25)	25.29 (0.62)
Cigarette smoking (%)			
Never	29.52	34.98	53.81
Former	26.36	31.81	17.78
Current (<20 cigarettes/day)	32.91	22.08	25.01
Current (20-40 cigarettes/day)	0.94	7.16	0.00
Current (e 40 cigarettes/day)	10.26	3.98	3.41
Alcohol intake (%)			
Up to once a week	53.87	47.29	52.57
2-3 times a week	0.00	18.14	19.46
4-6 times a week	28.54	17.23	12.89
Daily or more	17.58	17.34	15.08
Vigorous Physical activity (%)	12.49	13.59	18.11
Diabetes (%)	9.26	3.74	3.10
Calcium (mmol/L)			
Mean (SE)	1.13 (0.004)	1.23 (0.002)	1.32 (0.004)
Vitamin D (ng/mL)			
Mean (SE)	26.32 (2.17)	31.04 (0.69)	32.93 (1.45)
Albumin (g/L)			
Mean (SE)	42.75 (0.86)	43.22 (0.16)	43.74 (0.39)

Table 1 Age-adjusted (standardized to the 2000 US Census age distribution) weighted characteristics by quintiles of serum calcium, men, NHANES III 1988-1991.

	Geometric mean (95% confidence interval) of serum normalized calcium (mmol/L)			P for trend	P for T-test between lowest and top 5%
	Lowest 5% <1.16	**Mid 90% 1.16-1.30**	**Top 5% e1.30**		
Age and Race Adjusted Model					
Total testosterone (ng/ml)	4.86 (4.16-5.67)	4.98 (4.81-5.16)	5.30 (4.98-5.65)	0.031	0.010
Total estradiol (pg/ml)	36.86 (33.25-40.87)	35.95 (34.39-37.57)	32.41 (29.11-36.09)	0.197	0.302
SHBG (nmol/l)	35.60 (32.23-40.58)	34.39 (32.93-35.92)	39.89 (35.62-44.67)	0.011	0.383
Androstanediol glucuronide (ng/ml)	13.08 (9.64-17.74)	11.62 (11.04-12.23)	11.42 (9.98-13.05)	0.488	0.245
Free testosterone (ng/ml)	0.095 (0.085-0.107)	0.099 (0.096-0.103)	0.098 (0.090-0.106)	0.363	0.023
Free estradiol (pg/ml)	0.944 (0.866-1.029)	0.920 (0.875-0.968)	0.789 (0.699-0.891)	0.082	0.259
25-hydroxy Vitamin D (ng/mL)	23.27 (18.12-29.89)	29.12 (27.78-30.51)	31.85 (29.33-34.57)	0.072	0.050
Fully Adjusted Model					
Total testosterone (ng/ml)	4.75 (4.15-5.43)	4.98 (4.85-5.12)	5.20 (4.81-5.62)	0.441	0.551
Total estradiol (pg/ml)	35.03 (30.83-39.80)	36.01 (34.60-37.47)	32.23 (29.18-35.59)	0.158	0.163
SHBG (nmol/l)	36.36 (32.78-40.33)	34.16 (32.87-35.51)	38.94 (35.79-42.38)	0.006	0.212
Androstanediol glucuronide (ng/ml)	13.46 (10.28-17.63)	11.68 (11.05-12.35)	11.91 (10.15-13.98)	0.592	0.580
Free testosterone (ng/ml)	0.091 (0.080-0.103)	0.100 (0.097-0.103)	0.098 (0.089-0.108)	0.341	0.145
Free estradiol (pg/ml)	0.883 (0.783-0.995)	0.923 (0.883-0.965)	0.797 (0.717-0.887)	0.048	0.379
25-hydroxy Vitamin D (ng/mL)	23.52 (18.43-30.01)	29.16 (27.92-30.46)	32.40 (29.94-35.07)	0.038	0.045

Table 2 Geometric mean (95% CI) of sex steroid hormone concentrations by extreme measures of calcium in a nationally representative sample of adult men in NHANES III. All adjusted means take into account age, race/ethnicity, %body fat, diabetes, cigarette smoking, alcohol intake, vigorous physical activity, and serum levels of vitamin D and albumin.

	Geometric mean (95% confidence interval) of serum normalized calcium (mmol/L)			P for trend	P for Interaction
	Lowest 5% <1.16	Mid 90% 1.16-1.30	Top 5% e1.30		
Age 20-39					
Total testosterone (ng/ml)	5.64 (4.68-6.80)	5.71 (5.50-5.92)	6.27 (5.59-7.03)	0.292	
Total estradiol (pg/ml)	33.50 (26.32-42.63)	37.86 (36.55-39.22)	35.30 (31.08-40.09)	0.351	
SHBG (nmol/l)	36.00 (31.25-41.46)	30.01 (28.40-31.70)	36.13 (31.02-42.09)	0.003	
Androstanediol glucuronide (ng/ml)	14.94 (10.08-22.16)	12.98 (12.28-13.75)	13.60 (11.24-16.45)	0.726	
Free testosterone (ng/ml)	0.110 (0.092-0.131)	0.124 (0.120-0.128)	0.124 (0.111-0.138)	0.432	
Free estradiol (pg/ml)	0.833 (0.665-1.042)	0.990 (0.950-1.032)	0.874 (0.761-1.005)	0.092	
Vitamin D (ng/mL)	26.63 (21.85-32.47)	30.52 (28.67-32.50)	34.64 (29.65-40.47)	0.151	
Age 40-59					
Total testosterone (ng/ml)	4.22 (3.37-5.28)	4.61 (4.33-4.90)	4.59 (3.62-5.81)	0.793	0.564
Total estradiol (pg/ml)	34.81 (29.65-40.88)	34.69 (33.04-36.42)	29.09 (24.47-34.57)	0.195	0.586
SHBG (nmol/l)	33.31 (27.98-39.66)	35.05 (33.30-36.89)	37.51 (33.30-42.26)	0.447	0.398
Androstanediol glucuronide (ng/ml)	11.20 (7.67-16.36)	11.14 (10.25-12.10)	11.92 (7.18-19.79)	0.968	0.925
Free testosterone (ng/ml)	0.084 (0.068-0.104)	0.091 (0.085-0.097)	0.088 (0.067-0.117)	0.785	0.919
Free estradiol (pg/ml)	0.911 (0.767-1.083)	0.896 (0.851-0.943)	0.745 (0.617-0.117)	0.223	0.505
Vitamin D (ng/mL)	19.73 (13.18-29.54)	28.04 (26.87-29.25)	30.85 (23.73-40.05)	0.219	0.085
Age e60					
Total testosterone (ng/ml)	3.53 (2.86-4.36)	3.88 (3.56-4.22)	3.87 (2.75-5.43)	0.675	
Total estradiol (pg/ml)	33.66 (27.91-40.60)	33.45 (31.76-35.23)	29.19 (21.28-40.02)	0.671	
SHBG (nmol/l)	46.03 (40.19-52.72)	47.89 (45.81-50.06)	50.22 (36.27-69.55)	0.838	
Androstanediol glucuronide (ng/ml)	12.37 (6.75-22.69)	9.36 (8.62-10.17)	8.45 (5.55-12.87)	0.577	
Free testosterone (ng/ml)	0.057 (0.045-0.072)	0.062 (0.057-0.068)	0.059 (0.041-0.086)	0.744	
Free estradiol (pg/ml)	0.815 (0.690-0.964)	0.795 (0.753-0.840)	0.675 (0.493-0.923)	0.584	
Vitamin D (ng/mL)	25.87 (21.06-31.78)	27.66 (26.27-29.11)	24.57 (21.57-27.98)	0.228	

Table 3 Age-stratified geometric mean (95% CI) of sex steroid hormone concentrations by extreme measures of calcium in a nationally representative sample of adult men in NHANES III. All models are adjusted for age, race/ethnicity, %body fat, diabetes, cigarette smoking, alcohol intake, vigorous physical activity, and serum levels of vitamin D and albumin.

	Geometric mean (95% confidence interval) of serum normalized calcium (mmol/L)			P for trend	P for Interaction
	Lowest 5% **<1.16**	**Mid 90%** **1.16-1.30**	**Top 5%** **e1.30**		
Non-Hispanic white					
Total testosterone (ng/ml)	4.88 (4.17-5.71)	5.04 (4.85-5.23)	5.35 (4.76-6.00)	0.475	
Total estradiol (pg/ml)	35.14 (29.94-41.25)	35.57 (34.00-37.21)	32.43 (28.97-36.30)	0.402	
SHBG (nmol/l)	36.90 (32.11-42.40)	34.61 (33.17-36.11)	40.78 (36.31-45.81)	0.010	
Androstanediol glucuronide (ng/ml)	14.49 (10.66-19.71)	12.29 (11.49-13.15)	11.98 (9.96-14.40)	0.568	
Free testosterone (ng/ml)	0.093 (0.080-0.109)	0.100 (0.096-0.104)	0.098 (0.086-0.111)	0.638	
Free estradiol (pg/ml)	0.884 (0.763-1.024)	0.906 (0.862-0.952)	0.791 (0.699-0.894)	0.165	
Vitamin D (ng/mL)	24.53 (18.73-32.13)	31.57 (30.08-33.13)	34.97 (31.37-38.99)	0.057	
Non-Hispanic black					
Total testosterone (ng/ml)	3.93 (2.95-5.23)	5.49 (5.17-5.84)	5.46 (4.04-7.36)	0.034	0.091
Total estradiol (pg/ml)	34.24 (30.98-37.85)	42.94 (41.41-44.52)	35.13 (31.00-39.80)	<0.001	0.005
SHBG (nmol/l)	34.43 (26.74-44.32)	35.10 (34.00-36.86)	40.69 (33.68-49.15)	0.431	0.030
Androstanediol glucuronide (ng/ml)	10.73 (7.92-14.54)	10.50 (9.72-11.35)	13.27 (9.33-18.87)	0.496	0.655
Free testosterone (ng/ml)	0.072 (0.051-0.097)	0.111 (0.104-0.118)	0.103 (0.074-0.143)	0.010	0.384
Free estradiol (pg/ml)	0.864 (0.761-0.981)	1.110 (1.069-1.153)	0.872 (0.768-0.990)	<0.001	0.003
Vitamin D (ng/mL)	16.00 (9.48-27.00)	20.19 (18.10-22.53)	19.74 (17.20-22.65)	0.512	0.653
Mexican American					
Total testosterone (ng/ml)	4.74 (3.76-5.98)	5.17 (5.02-5.32)	4.79 (4.29-5.35)	0.300	
Total estradiol (pg/ml)	37.35 (30.75-45.38)	33.46 (32.20-34.76)	34.92 (31.73-38.45)	0.309	
SHBG (nmol/l)	26.67 (22.75-33.66)	31.12 (29.99-32.28)	25.25 (22.31-28.58)	0.004	
Androstanediol glucuronide (ng/ml)	12.74 (9.46-17.14)	11.53 (10.81-12.30)	10.50 (7.07-15.57)	0.601	
Free testosterone (ng/ml)	0.106 (0.086-0.132)	0.110 (0.106-0.113)	0.112 (0.097-0.129)	0.932	
Free estradiol (pg/ml)	1.019 (0.848-1.224)	0.881 (0.847-0.916)	0.968 (0.877-1.069)	0.023	
Vitamin D (ng/mL)	22.32 (17.77-28.04)	25.21 (24.62-25.82)	27.12 (24.72-29.76)	0.278	

Table 4 Race/ethnicity-stratified geometric mean (95% CI) of sex steroid hormone concentrations by by extreme measures of calcium in a nationally representative sample of adult men in NHANES III. All models are adjusted for age, race/ethnicity, %body fat, diabetes, cigarette smoking, alcohol intake, vigorous physical activity, and serum levels of vitamin D and albumin.

	Geometric mean (95% Confidence Interval) of serum normalized Calcium (mmol/L)			P for trend	P for interaction
	Lowest 5% **<1.16**	**Mid 90%** **1.16-1.30**	**Top 5%** **e1.30**		
Vitamin D < 24.2 ng/mL					
Total testosterone (ng/ml)	3.60 (3.00-4.31)	4.51 (4.24-4.80)	5.21 (4.43-6.13)	0.015	0.136
Total estradiol (pg/ml)	32.91 (29.64-36.55)	36.72 (35.21-38.29)	31.03 (28.71-33.54)	0.005	0.145
SHBG (nmol/l)	31.91 (29.17-34.90)	32.54 (31.47-33.64)	36.26 (30.60-42.96)	0.361	0.873
Androstanediol glucuronide (ng/ml)	10.65 (9.26-12.26)	10.97 (9.98-12.06)	11.53 (9.02-14.74)	0.834	0.468
Free testosterone (ng/ml)	0.072 (0.059-0.087)	0.092 (0.087-0.098)	0.103 (0.083-0.128)	0.022	0.232
Free estradiol (pg/ml)	0.864 (0.779-0.957)	0.959 (0.921-0.999)	0.792 (0.724-0.868)	0.004	0.135
Vitamin D e 24.2 ng/mL					
Total testosterone (ng/ml)	5.25 (4.55-6.07)	5.21 (5.08-5.35)	5.21 (4.79-5.67)	0.993	
Total estradiol (pg/ml)	36.76 (31.65-42.70)	35.68 (34.08-37.35)	32.71 (29.29-36.53)	0.325	
SHBG (nmol/l)	38.52 (33.31-44.54)	34.94 (33.04-36.95)	39.56 (34.97-44.74)	0.05	
Androstanediol glucuronide (ng/ml)	14.92 (10.41-21.39)	12.01 (11.28-12.79)	12.26 (10.50-14.32)	0.516	
Free testosterone (ng/ml)	0.098 (0.087-0.112)	0.103 (0.100-0.107)	0.096 (0.088-0.105)	0.323	
Free estradiol (pg/ml)	0.910 (0.791-1.045)	0.907 (0.859-0.957)	0.801 (0.708-0.907)	0.177	

Table 5 Vitamin D (median) -stratified geometric mean (95% CI) of sex steroid hormone concentrations by extreme measures of calcium in a nationally representative sample of adult men in NHANES III. All models are adjusted for age, race/ethnicity, %body fat, diabetes, cigarette smoking, alcohol intake, vigorous physical activity, and serum levels of vitamin D and albumin.

Figures

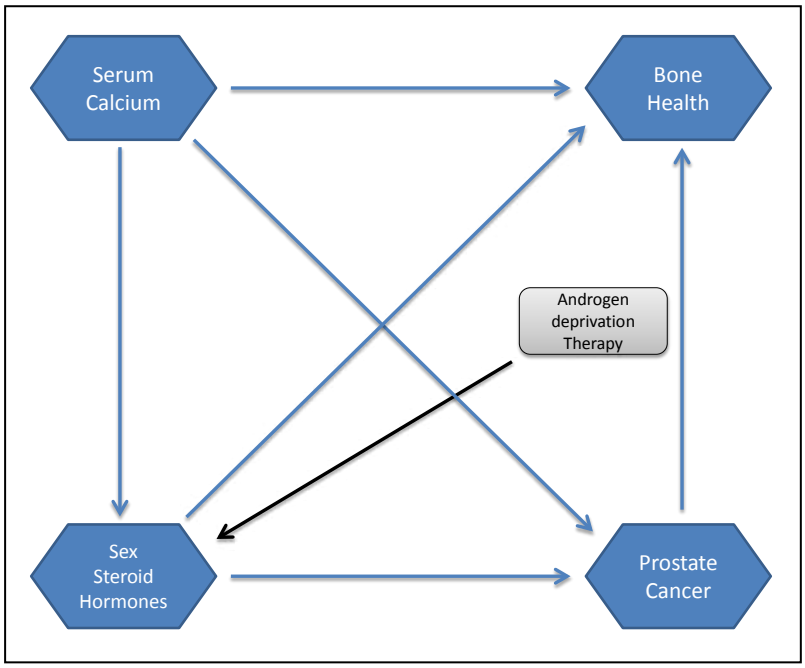

Figure 1 Potential pathways between serum calcium and sex steroid hormones.